学校编码：10384　　分类号＿＿＿密级＿＿＿
学号：K0426019　　UDC ＿＿＿＿＿＿＿＿

厦门大学

硕士学位论文

重组牛肠激酶活力影响因素及抑制动力学研究

The Study of Recombinant Enterokinase Activity Influence Factors and Their Inhibit Dynamics

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论文提交日期：2009 年 8 月 30 日
论文答辩时间：2009 年 10 月 31 日
学位授予日期：2009 年　月　日

答辩委员会主席：黄耀坚
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2009 年 11 月

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2009　年　11 月　3 日

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中文摘要

肠激酶（Enterokinase，EC 3.4.21.9，简称 EK）是存在于哺乳动物十二指肠内的一种异源二聚体丝氨酸蛋白酶，为激活胰蛋白酶原的关键酶，其催化亚基可以特异性识别 Asp-Asp-Asp-Asp-Lys 并沿其羧基端切下，将胰蛋白酶原活化为胰蛋白酶，从而启动各种酶原活化的级联。本文研究的肠激酶是酵母表达的 BEK (简称 BEK) 轻链接近电泳单一纯。主要通过催化荧光底物 Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide（简称 GD_4K-NA）水解跟踪法，部分项目结合 IL-11 融合蛋白水解产物进行电泳对该酶制剂的一些理化性质及活性影响方面进行研究，获得如下研究结果：

1．最适 pH 和 pH 稳定性：EK 催化 GD_4K-NA 水解反应的最适 pH 为 8.0。该酶在 pH 5-9 范围内较为稳定。超过这个 pH 范围时，酶的活力迅速降低。

2．最适温度和温度稳定性：酶催化反应的最适温度为 37℃左右，在 10~37 ℃范围内活性与温度呈正相关，温度超过 40 ℃后，酶的耐热特性明显下降，活力迅速降低。在 50 ℃以上不适于酶活的测定。-20 ℃最适于 BEK 的长期保存。

3．氨基酸对酶活力的影响效应：考察它们对 BEK 的浓度效应，发现 L-Gly，L-Ala、L-Ile、L-Pro、L-Val、L-Leu、L-Phe、L-Met、L-Ser、L-Thr、L-Cys、L-Asp 和 L-His 对酶活力没有明显作用；L-Glu 对酶活力有一定的浓度效应；L-Lys、L-Arg 对酶活力有较大的影响，当浓度为 30 mmol/L 时，L-Lys、L-Arg 分别可使酶活力下降 60%、43%, 求得 L-Lys 的 IC_{50} 为 25 mmol/L。L-Lys、L-Arg 对 EK 酶的抑制作用均属于可逆过程，抑制类型表现为竞争性类型，两者对 EK 酶抑制常数 K_I 分别为 12.02 和 35.14 mmol/L。

4．有机溶剂的影响：乙醇对酶活力有一定的促进作用，甲醇对 BEK 的酶活力没有影响，而正丙醇、乙腈、丙酮对 BEK 具有一定的抑制作用，其半抑制浓度（IC_{50}）分别为 2.8 mol/L，3.2mol/L 和 50 mmol/L，丙酮对 BEK 的抑制属于不可逆过程。

5．EDTA、DTT 等对 BEK 活力的影响：EDTA 与 DTT 均对 BEK 具有抑制作用，两者的 IC_{50} 分别为 50 和 120 mmol/L。

6．变性剂对酶的浓度效应及抑制类型：脲素及盐酸胍对 EK 的抑制作用的 IC_{50} 分别约为 0.5mol/L 和 25 mmol/L。发现合成的缩氨基硫脲对 EK 的抑制作用表现为可逆效应，抑制类型为混合型，测得 4-羟基苯甲醛缩氨基硫脲对 EK 的抑制常数 K_I 及对酶-底物络合物（ES）的抑制常数 K_{IS} 分别为 64.3 和 226.4 μmol/L。

7．金属离子的影响：K^+对酶活力没有明显的影响，Mg^{2+}对酶活力有一定的影响，Ca^{2+}、Zn^{2+}对 EK 酶的抑制作用较强，导致酶活力下降 50%所需的 Ca^{2+}、Zn^{2+} 浓度（IC_{50}）分别为 3.2 和 0.9 mmol/L。Zn^{2+}对酶的抑制属于不可逆过程。

8．建立了商品化 EK 的测活模型。通过荧光底物跟踪法快速地对工业化生产 EK 活性进行测定。对各个单位的生产的不同定义的酶活力进行比较，建立联系和统一评价标准。

关键词：活性测定；牛肠激酶；动力学；抑制作用

Abstract

Enterokinase（EC 3. 4. 21. 9，EK）is a heterologous dimer serine protease esixsting in mammals' duodenal. It is the key enzyme in specifically cleaves trypsinogen by recognizing Asp-Asp-Asp-Asp-Lys at the carboxyl terminal and turn it into active trypsin to start the cascade of a variety of proenzyme activation. The purified enzyme investigated in this acticle was nearly a single band on SDS-PAGE. The main method in this study was to track the rate of catalyze hydrolysis the fluorescent substrate GD_4K-NA, some investigation was combinated with the method of electrophoresis the hydrolysis product of the enzyme cleavage IL-11 fusion protein to study the physico-chemical property of the enzyme preparation and the influence factor for the activity of the enzyme. The result had been obtained as following:

1) The optimum pH value of EK hydrolysis GD_4K-NA is 8.0 and the enzyme is stability at the range of 5.0~9.0, when the pH out of the range, the activity of the enzyme will be suppressed quickly.

2) The optimum temperature for enzyme is about 37℃, at the range of 10℃ to 37℃, the activity of the enzyme is positive correlation to temperature, when the temperature more than 40℃, the enzyme stability will dramatic decline, reflect the activity quickly decrease. Beyond 50 ℃ is not suitable for enzyme activity determined and -20℃ is optimum to BEK long-term preservation.

3) The effects of some amino acids on the enzyme activity were studied. The results showed that the inhibitory effects of non-polar amino acids such as L-Gly, L-Ala, L-Ile, L-Pro, L-Val, L-Leu, L-Phe, L-Met, L-Ser, L-Thr, L-Cys, L-Asp and L-His had no significant effects; L-Glu had some concentration dependent inhibitory effect on the enzyme; L-Lys and L-Arg had inhibitory effects on the enzyme activity. When the concentration of inhibitor were 30 mmol/L, L-Lys and L-Arg would cause the enzyme activity descended 60% and 43%, respectively, The IC_{50} of L-Lys was 25 mmol/L. The inhibitory effects of L-Lys and L-Arg were reversible with remaining enzyme activity and the inhibitory mechanisms were tested to be competitive types and their K_I were determined to be 12.02 and 35.14 mmol/L, respectively.

4) The effect of organic solvents on the enzyme were investigated. The results of research showed that alcohol had some promotion on the enzyme，methanol had no inhibitory effect, while acetone, N-propanol and acetonitrile had some inhibitory effect on the enzyme, the IC_{50}s of them were 2.8 mol/L, 3.2 mol/L and 50 mmol/L, respectively. The inhibitory effect of acetone was irreversible.

5) The inhibitory effect of DTT and EDTA on activity of BEK were investigaged. The results of research showed that DTT and EDTA have inhibitory effect, the IC_{50}s of EDTA and DTT were 50 and 100 mmol/L.

6) The inhibitory effect of denaturant on the enzyme: the IC_{50}s of urea and guanidine hydrochloride were about 0.5 mol/L and 25 mmol/L. Some kinds of Synthesis thiosemicarbazone had reversible inhibitory effects on the enzyme and the inhibitory mechanisms were tested to be mixed type inhibition, for the example of 4-hydroxybenzaldehyde thiosemicarbazone, the inhibition constant to the enzyme (K_I) and to the Enzyme-substrate complex (K_{IS}) were determined to be 64.3 and 226.4 μmol/L, respectively.

7) The metal ions had dissimilar effects to the activity of the enzyme. K^+ didn't affect the activity of BEK, Mg^{2+} had inhibitory effect to some extent. While Ca^{2+} and Zn^{2+} had strong inhibitory effect to the activity of BEK, the IC_{50}s of them were 3.2 and 0.9 mmol/L. The inhibitory effect of Zn^{2+} was irreversible.

8) Establish activity determined model for commercial enterokinase by tracking the relative fluorescence increase rate to test the activity of industrial production EK quickly. Compare the definition EK activity unit between different manufacturer to establish the unified evaluation criteria.

Key words： activity assay; bovine enterokinase; dynamics; inhibitory

缩略词

缩略词	英文	中文
GD_4K-β- NA	Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide	甘氨酸-（天冬氨酸）$_4$-赖氨酸-β 萘胺
BEK	Bovine enterokinase	BEK
SDS-PAGE	Sodium dodcyl sulfate polyacrylamide gel electrophoresis	十二烷基硫酸钠—聚丙烯酰胺凝胶电泳
Marker	Low molecular marker of protein	低分子量蛋白标准
IC_{50}	The inhibitor concentrations leading to 50% activity lost	半抑制率浓度
K_m		酶促反应的表观米氏常数
Vmax(V_m)		酶促反应的最大反应速度
Arg	Arginine	精氨酸
Leu	leucine	亮氨酸
Lys	Lysine	赖氨酸
Phe	Phenylalanine	苯丙氨酸
Glu	Glutamic acid	谷氨酸
Asp	Aspartic acid	天冬氨酸
Thr	Threonine	苏氨酸
Met	Methionine	甲硫氨酸
Ile	Isoleucine	异亮氨酸